Bulletin of the World Health Organization, 65 (2): 181-187 (1987)

Serological studies with influenza A(H1N1) viruses cultivated in eggs or in a canine kidney cell line (MDCK)

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Pairs of influenza A(H1N1) viruses cultivated from the same clinical specimen in canine kidney (MDCK) cells or in embryonated hens' eggs can frequently be distinguished by their reactions with monoclonal antibodies to haemagglutinin and with antibodies in ferret or human sera. Egg-adapted virus, further passaged in MDCK cultures remained "egg-like" in serological characteristics indicating that the differences in their serological reactions were not a direct result of host cell-dependent glycosylation of the haemagglutinin. Haemagglutination-inhibiting (HI) or virus neutralizing antibodies in human sera can be detected more frequently, and to higher titre, in tests employing virus grown exclusively in MDCK cells than in tests with virus adapted to growth in embryonated eggs. Striking differences were detected in the serological reactions in HI tests when sera from ferrets infected with egg-grown virus were tested against a series of strains of influenza A(H1N1) virus isolated in 1983 and adapted to growth in eggs. In contrast, sera from ferrets infected with MDCK-derived virus failed to distinguish serologically between the same viruses that had been passaged exclusively in MDCK cells and also revealed relatively small differences between their egg-adapted counterparts.

It was concluded that the cell substrate used for virus isolation and cultivation is a factor that should be considered when interpreting the results of strain characterization of influenza A(H1N1) isolates and in sero-surveys using these viruses.

Antigenic analysis of influenza A and B virus strains is most commonly carried out using virus cultivated in embryonated hens' eggs (*1*). Although human influenza viruses were first isolated in an experimental animal, the ferret (*2*), and soon afterwards adapted to mice, extensive laboratory work only became possible with the observation that viruses could be isolated in the amniotic cavity of embryonated hens' eggs (*3*). In the absence of tissue culture systems at that time, the embryonated hens' eggs became the established method for cultivating influenza viruses. Subsequently Burnet demonstrated that human isolates of influenza virus were mixtures of variants with differing biological properties and that the complex environment of the amnion or allantoic cavity of the egg exerted differing selective pressures (reviewed in *4*). Additional selective pressures that could result in a degree of separation of variants include naturally occurring alpha, beta and gamma inhibitors (*5*) and, of course, specific antibody to the virus itself (*6*). More recently we have presented evidence for the host-cell selection of influenza B virus (*7*). Viruses isolated from clinical specimens and serially cultivated in mammalian cells (a canine kidney cell line, MDCK), possessed haemagglutinins (HA) that were antigenically distinguishable from those of viruses grown from the same source but in embryonated hens' eggs. Differences in the amino acid sequence of the HA of influenza B virus cultivated in MDCK cells and in eggs have been identified (*8*). We concluded that adaptation of influenza B virus to growth in eggs selected a virus subpopulation (*4*, *6*, *9-11*) which was antigenically and biochemically distinct from virus from the same clinical source but passaged exclusively in MDCK cells.

In the present paper we describe marked differences in the serological characteristics in both haemagglutination inhibition (HI) and virus neutralization tests with monoclonal anti-HA antibodies of influenza A(H1N1) viruses isolated from the same

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clinical specimens in MDCK cells or in eggs. Moreover, in HI or virus neutralization tests with human and ferret sera, virus cultivated exclusively in MDCK cells detected antibody more frequently and at higher titre than the corresponding egg-adapted virus. Our findings suggest that interpretation of serological and antigenic analyses of influenza A(H1N1) viruses, like those for influenza B viruses (*7*), may be complicated by selection of antigenic variants during virus cultivation in different host-cell systems. The observations may also have significance for our understanding of the antigenic structure and variation of influenza virus haemagglutinin (*12*) and immune responses to infection and immunization.

MATERIALS AND METHODS

Virus isolation

The influenza A viruses studied were isolated from an influenza outbreak in a residential school, Christ Hospital School, in February 1983 (40 strains designated A/Chr/83), and from Florence (2 strains). Most viruses were isolated in Madin–Darby Canine Kidney Cell (MDCK) cultures and further passaged in these cells maintained in Eagle's minimal essential medium containing TPCK trypsin (0.2 μg/ml). For isolation of virus in eggs, 0.1 ml of a virus suspension from a throat swab or from a primary isolation in MDCK cells was inoculated into the amniotic cavities of eleven-day old embryonated hens' eggs. After incubation at 33 °C, for 48 hours, the allantoic and amniotic fluids were harvested, tested for the presence of virus, and stored at −70 °C ("egg-virus"). Egg-adapted virus isolates were further passaged twice in the allantoic cavity and the allantoic fluids used as antigen for serological H1 tests.

Monoclonal antibodies and serological tests

Monoclonal antibodies to the HA of A/USSR/92/77, A/Chr/91/83, A/Brazil/11/78, A/England/333/80 and A/Baylor/5700/82 (H1N1) viruses were prepared using standard procedures (*13*, *14*). In brief, mice were immunized twice with purified influenza A virus prepared in eggs and spleen cells removed 4 days after the second booster dose of antigen, which was given intravenously. Antibody was prepared as mouse ascitic fluids and used for the HI tests after overnight treatment at 37 °C with 4 volumes of receptor-destroying enzyme[a] to remove any non-specific inhibitors. The micro-HI test with 96-well microtitration plates[b] was used and the challenge dose of virus was carefully standardized by repeat titration to 8 HA units. In each experiment the homologous viruses were included as a control and reproducibility of HI titres was established by experiment.

Single radial haemolysis was carried out as described previously (*15*). Neutralization experiments were carried out by mixing approximately 100 $TCID_{50}$ of virus with varying dilutions of monoclonal antibody or human serum for 1 hour at 37 °C and testing the resultant antibody–virus mixtures for residual infective virus in MDCK cells.

Post-infection human and ferret sera

Sera were obtained from non-immunized children and adults (aged 2–45 years) in the United Kingdom in November 1983. The antibody detected in the sera was assumed to result from natural influenza A(H1N1) infection during or after the time the viruses were isolated at Christ Hospital School. Ferret sera were obtained from animals infected intranasally with egg-grown or MDCK cell-grown influenza A/Chr/157/83 (H1N1), A/Brazil/78 or A/Chile/1/83 viruses and bled 10 days later.

RESULTS

Antigenic analysis of MDCK-cell-grown and egg-grown influenza A(H1N1) viruses using a panel of anti-HA monoclonal antibodies

Results of HI tests on nine representative influenza A(H1N1) viruses adapted to growth in eggs or cultivated exclusively in MDCK cells are presented in Table 1. Marked differences were noted in the HI reactions of the viruses against a large panel of monoclonal antibodies dependent upon the passage histories of the viruses. In all, 63 viruses were tested; the majority, 53 of them, could be distinguished antigenically when corresponding viruses adapted to growth in eggs or cultivated exclusively in MDCK cells were compared.

Three patterns of serological reactions were discerned for the monoclonal antibodies. Three of the twelve antibodies reacted exclusively with egg-adapted virus (Br29, E23 and B3). In contrast, other antibodies (E61 and B7) reacted with virus cultivated exclusively in MDCK cells but not egg-adapted virus for some isolates, and for other isolates they reacted with virus grown in both substrates. A third group of antibodies reacted equally well with both egg-adapted and MDCK-cell-derived virus (e.g., Br2). Egg- or MDCK-derived A/Chr/157/83 and A/Chr/91/83 viruses, used in the subsequent serological studies with human and ferret sera (described below), were clearly distinguished with monoclonal antibodies E61, B3 and B7.

[a] From Philips, Duphar B. V., Amsterdam, Netherlands.

[b] Linbro plates supplied by Flow Laboratories, Woodcock Hill, Herts., England.

Table 1. Serological analysis of MDCK-cell-grown and egg-grown influenza A(H1N1) viruses using monoclonal antibodies to HA

Virus and host cell used for cultivation[a]		Viruses and monoclonal antibodies										
		A/USSR/0092/77			A/Brazil/11/78		A/England/333/80			A/Baylor/5700/82		
		U22	U70	UW18	Br2	Br29	E23	E58	E61	B1	B3	B7
A/USSR/0092/77	E	1600	6400	3200	>12800	>12800	6400	<100	1600	800	<100	<100
A/Brazil/11/78	E	1600	12800	1600	>12800	6400	3200	800	1600	1600	<100	400
A/India/6263/80	E	800	6400	100	6400	3200	400	400	1600	<100	<100	<100
A/England/333/80	E	1600	6400	800	6400	3200	6400	800	3200	1600	<100	<100
A/England/403/80	E	1600	6400	<100	6400	3200	3200	400	1600	800	<100	1600
A/Hong Kong/2/82	E	1600	3200	400	>12800	6400	<100	800	1600	1600	<100	800
A/Dunedin/27/83	E	<100	<100	<100	3200	100	<100	200	<100	3200	12800	800
A/Chr/892/83	C	<100	<100	200	3200	<100	<100	800	1600	1600	<100	6400
	E	<100	<100	<100	3200	<100	<100	400	<100[b]	3200	<100	<100
A/Chr/920/83	C	<100	<100	200	3200	<100	<100	800	1600	1600	<100	3200
	E	400	400	400	6400	1600[b]	1600	1600	3200	800	<100	<100
A/Chr/922/83	C	<100	<100	200	3200	<100	<100	800	1600	1600	<100	6400
	E	100	<100	<100	6400	<100	<100	400	<100	6400	12800	1600
A/Chr/965/83	C	<100	<100	200	3200	<100	<100	400	1600	1600	<100	6400
	E	100	<100	400	3200	800	3200	400	3200	1600	<100	<100
A/Chr/83/83	C	<100	<100	200	3200	<100	<100	800	1600	1600	<100	3200
	E	<100	<100	<100	3200	<100	<100	800	<100	<100	<100	<100
A/Chr/91/83	C	<100	<100	200	3200	<100	200	400	1600	1600	<100	3200
	E	<100	<100	<100	3200	<100	<100	200	<100	3200	12800	<100
A/Chr/157/83	C	<100	<100	200	3200	<100	400	800	1600	1600	<100	3200
	E	<100	<100	<100	3200	<100	<100	1600	<100	3200	12800	<100
A/Florence/13/83	C	800	1600	<100	12800	NT[c]	<100	NT	1600	800	<100	NT
	E	1600	12800	<100	12800	NT	<100	NT	<100	1600	<100	NT
A/Florence/19/83	C	400	800	200	3200	NT	<100	NT	1600	400	<100	NT
	E	400	3200	200	6400	NT	1600	NT	1600	400	<100	NT

[a] C = MDCK cell; E = allantoic cavity of embryonated hens' eggs.

[b] The underlined titres indicate significant serological differences between cell- and egg-grown virus.

[c] NT = not tested.

A striking finding was that very few strain-to-strain serological differences were observed between different viruses that had been cultivated exclusively in MDCK cells (Table 1). In contrast, the different isolates that had been adapted to growth in eggs were heterogeneous in their serological reactions with individual monoclonal antibodies. For example, all nine MDCK-derived isolates reacted with monoclonal antibody E61 but only three of the nine egg-adapted viruses isolated from the same clinical specimens reacted with this antibody.

To investigate these differences further, certain viruses were examined serologically using ferret sera from animals infected with either egg-adapted virus or virus cultivated exclusively in MDCK cells. Firstly, it is apparent (Table 2) that sera such as F10/84 or F3/85, from animals infected with MDCK-cell-derived virus, generally do not distinguish serologically between the isolates such as A/Chr/157/83, A/Chr/91/83 and A/Chr/83 regardless of whether the viruses are cultivated in MDCK cells or in eggs. In contrast, in an HI analysis using sera from ferrets infected with egg-derived virus, antigenic differences are detected among this group of isolates, particularly if the viruses used in the HI test are cultivated in eggs. Thus, serum F6/84 had HI titres of 960 and 20, respectively, when reacted with egg-derived A/Chr/157/83 and A/Chr/83/83 viruses in

Table 2. HI reactions of post-infection ferret sera with influenza A(H1N1) viruses cultivated exclusively on either MDCK cells or eggs

Virus and host cell used for cultivation[a]		HI titre with following ferret sera: Ferrets infected with MDCK-derived A/Chr/157/83 virus F10/84	F3/85	Ferrets infected with egg-derived A/Chr/157/83 virus F6/84	F6/85	Ferrets infected with egg-derived A/Brazil/78 virus	A/Chile/1/83 virus
A/Chr/157/83	C	480	3200	80	240	80	480
A/Chr/91/83	C	640	4800	120	240	40	480
A/Chr/892/83	C	640	4800	120	240	40	480
A/Chr/922/83	C	640	3200	120	240	–	–
A/Chr/83/83	C	640	3200	120	240	40	480
A/Chr/157/83	E	320	800	960	>2560	20	60
A/Chr/91/83	E	480	800	480	>2560	40	120
A/Chr/892/83	E	480	1600	320	640	30	240
A/Chr/922/83	E	480	1200	480	>2560	–	–
A/Chr/83/83	E	160	600	20	<20	<20	40
Reference viruses:							
A/USSR/92/77	E	75	200	<20	<20	480	160
A/Brazil/11/78	E	150	1200	<20	50	640	240
A/India/6263/80	E	600	1200	75	150	80	480
A/Chile/1/83	E	200	1200	25	50	40	320

[a] C = MDCK cell; E = hens' eggs.

Table 3. Haemagglutination inhibition (HI) and virus neutralization titres of influenza A/Chr/91/83 (H1N1) virus derived exclusively from MDCK cells or passaged in eggs

Antibody	Serological reactivity with egg- or cell-derived virus	HI titre: MDCK-cell-derived virus (M6)[a]	Egg-derived virus (M1E5)[a]	Egg-derived virus cultivated in MDCK cells (M1E5M1)[a]	Neutralization titre: MDCK-cell-derived virus (M6)	Egg-derived virus (M1E5)	Egg-derived virus cultivated in MDCK cells (M1E5M1)
Monoclonal antibody							
Br2	Both	3 200	3 200	NT[b]	16 000	8 000	NT
E61	Cell	1 600	<100	<100	6 000	<500	<500
E336	Cell	12 800	<100	<100	22 000	<500	<500
B3	Egg	<100	12 800	12 800	<500	64 000	⩾64 000
B11	Egg	<100	6 400	6 400	<500	6 000	20 000
Post-infection human serum[c]							
1	–	960	<10	<10	2 400	30	<10
2	–	120	<10	<10	120	15	<10
3	–	240	<10	<10	240	15	<10
4	–	120	<10	<10	320	30	<10

[a] Number of passages indicated in MDCK cells (e.g., M6) and in embryonated hens' eggs (e.g., E5).

[b] NT = not tested.

[c] Sera collected in November 1983.

the HI test, whereas the same serum failed to distinguish between the same two viruses when they were cultivated in MDCK cells (HI titres of 80 and 120, respectively).

The viruses with different passage histories were also clearly distinguishable in neutralization tests with the same monoclonal antibodies as used in the HI reaction (Table 3). For example, monoclonal antibody E61 neutralized only MDCK-cell-grown virus, whereas antibody B3 neutralized only egg-grown virus. Several of the monoclonal antibodies neutralized viruses grown in both substrates (e.g., Br2).

In further experiments egg-grown virus passaged subsequently in MDCK cells showed identical serological reactivity with the monoclonal and polyclonal antibodies (see below) as did virus cultivated exclusively in eggs (Table 3). Therefore, the differences detected above in serological reactions were not a manifestation of host-dependent glycosylation of the HA.

Electron microscopy of negatively-stained preparations of influenza A/Chr/91/83 (H1N1) virus cultivated in MDCK cells or in eggs failed to detect obvious morphological differences or stages of aggregation which could otherwise provide an explanation of the differing serological reactions.

Serological reactivity of human sera with influenza A(H1N1) viruses of different passage history

A total of 419 sera from individuals ranging from 2 to 65 years of age were examined using the HI test and viruses which had been grown exclusively in MDCK cells, or in eggs, or which had been adapted to growth in eggs and subsequently passaged in MDCK cells. The data pertaining to a representative 94 sera are shown in Table 4. It was assumed that the antibody in the human sera was generated by natural infection: the sera were taken in the months following the 1983 epidemic. The MDCK-cell-derived influenza A(H1N1) virus A/Chr/91/83 detected HI antibody at considerably higher frequency and titre in the sera than did the corresponding virus adapted to growth in eggs. A/Chr/91/83 virus cultivated exclusively in MDCK cells reacted with 65% of sera with an HI titre of $\geqslant 1/20$, whereas the same virus cultivated in eggs detected antibody in 20% of the same sera (Table 4).

Egg-adapted virus which was subsequently cultivated in MDCK cultures reacted with 16% of sera and thus was similar to the egg-grown virus. In addition, exclusively MDCK-cell-grown virus detected higher HI titres than egg-grown virus and, for example, 30% and 4% of sera had titres $\geqslant 60$ when tested using MDCK-cell-grown and egg-grown virus respectively. Essentially identical data were obtained when a group of 212 sera from younger individuals (2–11 years old) was tested against exclusively MDCK-cell or egg-grown A/Chr/157/83 (H1N1) virus and also when a group of 60 sera from older individuals (50–65 years old) was tested against exclusively MDCK-cell and egg-grown A/Chr/959/83 (H1N1) virus (data not presented).

Similarly, neutralizing antibody was detected more frequently and to higher titres using exclusively MDCK-cell-derived virus in the test than using egg-adapted virus. Egg-adapted virus subsequently passaged in MDCK cells reacted similarly to virus grown exclusively in eggs (Table 4). Thus 70% of sera had neutralizing antibody titres of $\geqslant 20$ when analysed using MDCK-cell-grown virus in the assay, whereas only 7% and 10% of sera had neutralizing antibody to egg-grown virus and egg-grown virus cultivated in MDCK cells, respectively.

In preliminary experiments absorption studies were performed on the adult sera to confirm the specificity

Table 4. Frequency and titre of antibody in human sera to A/Chr/91/83 (H1N1) virus passaged exclusively in MDCK cells or adapted to growth in eggs

Origin and passage history of virus[a]	Cumulative number of sera (out of 94 tested)[b] with the following HI titres:				Cumulative number of sera (out of 30 tested) with the following virus neutralization titres:			
	<20	⩾20	⩾60	⩾120	<20	⩾20	⩾60	⩾120
MDCK (M6)	33(35.1)[c]	61(64.9)	28(29.8)	10(10.6)	9(30.0)	21(70.0)	7(23.3)	3(10.0)
Egg (M1E5)	75(79.8)	19(20.2)	4(4.3)	0	28(93.3)	2(7.0)	0	0
Egg and MDCK (M1E5M1)	79(84.0)	15(16.0)	3(3.2)	0	27(90.0)	3(10.0)	0	0

[a] Number of passages indicated in MDCK cells (e.g., M6) and in hens' eggs (e.g., E5).

[b] Sera collected in the United Kingdom in 1983.

[c] Figures in parentheses are percentages.

of reaction with MDCK virus noted above. Ten sera from non-immunized adults whose antibodies were presumably induced by natural infection were absorbed with purified A/Chr/157/83 (H1N1) virus cultivated either in MDCK cells or in eggs and residual antibody investigated using the single radial haemolysis (SRH) technique with MDCK-cell-grown or egg-grown virus in the immunoplate. When the absorbed sera were tested on the SRH plate containing egg-grown virus, no residual antibody was detected. In contrast, when the same sera were tested on the SRH plate containing MDCK-cell-grown virus, haemolysis zones were present after the sera had been adsorbed with egg-grown virus, whereas adsorption with MDCK-cell-grown virus removed all antibody.

DISCUSSION

Antigenic differences were frequently observed between the haemagglutinins of influenza A(H1N1) viruses that had been isolated from the same clinical sample and cultivated exclusively in MDCK cells or adapted to egg growth. In addition, the viruses grown in different substrates could be clearly distinguished in virus neutralization tests with the monoclonal antibodies. Amino acid sequence changes have been detected in the haemagglutinins of influenza A(H1N1) viruses cultivated in eggs or MDCK cells in the present study. They are located on the surface of the globular head of the haemagglutinin molecule adjacent to the receptor binding site (J. Robertson et al, unpublished data).

The antigenic differences that have been elucidated here between the haemagglutinins of MDCK-cell and egg-grown influenza A(H1N1) viruses with monoclonal antibodies have been confirmed using polyclonal human and ferret sera. With human sera, both a higher frequency of antibodies and higher HI and virus neutralization titres were detected to MDCK-cell-grown virus compared to egg-grown influenza A(H1N1) virus.

A qualification of the serological data is that neutralization tests were carried out using only MDCK cells and it is possible that similar experiments when carried out in embryonated hens' eggs may give different results. Such experiments are in progress.

The serological reactivities of cell- and egg-grown viruses were independent of the last substrate in which the virus was cultivated. Both egg-grown virus and egg-grown virus cultivated in MDCK cells had identical serological characteristics with monoclonal and polyclonal antisera and, antigenically, were distinguishable from the exclusively MDCK-cell-grown virus: thus there could not be a direct result of the host cell of origin of carbohydrate side-chains of the haemagglutinin molecule. Finally, adsorption studies have demonstrated the presence of antibodies in human sera which react exclusively with MDCK-cell-grown virus and not with egg-grown virus.

The study has implications for the interpretation of results of sero-epidemiological studies with influenza A(H1N1) viruses, since both the HI and neutralization tests are at present routinely performed using egg-grown virus (reviewed in *1* and *16*). In the light of the antigenic differences between the egg- and cell-grown virus populations reported here, comparative sero-surveys which investigate the antibody to influenza A(H1N1) viruses grown in MDCK cells and eggs would seem to be of interest to determine which of these two methods of cultivation would be of greater value in an epidemiological context. Finally, experiments are in progress to investigate the implications of our findings for the design of influenza vaccines.

ACKNOWLEDGEMENTS

A. Douglas and J. J. Skehel, National Institute for Medical Research, Mill Hill, London, generously provided certain of the monoclonal antibodies used in the present study. J. Smith and E. Grilli (PHLS Laboratories, Guildford) kindly provided the viruses isolated at Christ Hospital School.

RÉSUMÉ

ÉTUDES SÉROLOGIQUES RÉALISÉES AVEC LE VIRUS GRIPPAL A(H1N1) CULTIVÉ SUR ŒUFS OU EN LIGNÉES DE CELLULES RÉNALES DE CHIEN (MDCK)

On peut fréquemment distinguer des virus grippaux A(H1N1) cultivés à partir du même prélèvement clinique en cellules rénales de chien (MDCK) ou en œufs de poule embryonnés par leur réaction avec des anticorps mono-

clonaux antihémagglutinine et avec des anticorps de sérum de furet ou de sérum humain. Les virus avianisés, après passage ultérieur en culture de MDCK, conservent leurs caractéristiques sérologiques initiales (de type "œuf"), ce qui montre que la différence de réactivité sérologique ne résulte pas directement de la glycosylation de l'hémagglutinine effectuée par la cellule hôte. Les anticorps neutralisants ou les anticorps inhibant l'hémagglutination (HI) présents dans le sérum humain peuvent être décelés plus souvent, et à un titre supérieur, par des épreuves utilisant des virus cultivés exclusivement en cellules MDCK que lors d'épreuves exécutées avec des virus adaptés à la réplication en œufs embryonnés. On a observé des différences frappantes en HI lorsqu'on a éprouvé, par comparaison avec une série de souches de virus grippal A(H1N1) isolées en 1983 et avianisées, du sérum de furets infectés par des virus cultivés sur œuf. En revanche, le sérum de furets infectés par un virus cultivé sur MDCK ne permettait pas d'établir une distinction sérologique entre les mêmes virus cultivés exclusivement sur cellules MDCK et ne révélait que des différences relativement faibles entre leurs homologues avianisés.

On peut conclure de cette étude que le substrat cellulaire utilisé pour l'isolement et la culture du virus est un facteur dont il faut tenir compte lorsqu'on interprète les résultats de la caractérisation des souches de virus grippal A(H1N1) et lors des enquêtes sérologiques utilisant de tels virus.

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